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- USSR -

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INCORPORATION OF FORMATE-C^{14} INTO NUCLEOPROTEIDS AND ALBUMINS OF EXPERIMENTAL TUMORS

- USSR -

[Following is a translation of an article by V. A. Kirsanova and Yu. S. Gurevich-Usyskina of the biochemistry laboratory of the Institute of Experimental Pathology and Therapeutics of Cancer, USSR Academy of Medical Sciences, Moscow. The article appeared in the Russian-language periodical Voprosy Meditsinskoy Khimii (Problems of Medical Chemistry), Moscow, Vol. VI, No. 3, 1960, pages 254-259.]

In studying combined chemotherapy of experimental tumors, interest was aroused in comparing the inclusion of formate-C^{14} in vivo and in vitro into nucleic acids (NA), and in our discovered active labile protein fraction of ascites cells, of a solid Ehrlich's tumor and a Crocker sarcoma. Many studies /1-4/ were devoted to the biosynthesis of NA in ascites cells, but they did not sufficiently examine the incorporation of tagged fore-runners in the protein fraction of nucleoproteids or the authors were dealing with a fraction different from that which we encountered in experiments with ascites cells [5].

METHOD OF INVESTIGATION

For comparing the inclusion of formate-C^{14} into ascites cells and the solid tumor we used normal white mice of 18-22 g. One group of animals was given 0.2 ml of ascites intraperitoneally, the other - subcutaneously. Experiments with ascites began after 6-8 days following injection, with subcutaneous humor - after 14 days. The experiment was performed on 300 mice.

In in vivo experiments formate-C^{14} was introduced into mice three hours before the experiment in a dose of 10,000 imp/sec per gram of body weight, and in in vitro experiments - 40,000 imp/min per gram of gross weight of ascites cells or tumor tissue. Tumors or ascites cells were taken from 4-8 mice for analysis. In in vitro experiments the ascites were collected in small ice-cooled flasks or centrifuge tubes. Heparin solution, diluted 40 times (the basic heparin solution contained 5,000 units/ml) was used to avoid coagulation in the syringe (0.02 ml solution per ml of ascites). Ascites cells were separated by centrifuging, washed twice in ice-cold physiological solution and weighed. In some experiments 8 ml of centrifuged ascitic fluid was added to one gram of cells, in other experiments

- 8 ml of Krebs-Ringer phosphate buffer, pH 7.4, to which were added 0.25 ml of a 4% solution of nicotinic acid amide and 40% solution of glucose or 0.4 ml of mouse liver homogenate prepared by the method of Schulman et al. /6/. (Buffer composition: 100 parts 0.9% NaCl, 4 parts 1.15% KCl. 3 parts 1.22% anhydrous $CaCl_2$, 1 part 2.11% KH_2PO_4, 1 part 3.82% $MgSO_4.7H_2O$ and 2 parts 0.1 M solution of phosphate buffer pH 7.4, 17.8 g $Na_2HPO_2.2H_2O$, and 20 ml 1N HCl solution in 1 liter distilled water.) Then formate and water to 10 ml were added, following which the suspensions were incubated with constant agitation on a water thermostat at 37° under aerobic conditions.

In the first tests incubation was continued for 2 hours, in later tests for 1 hour since it was discovered the formate is intensively incorporated into ascites cells after fifteen minutes following its addition, and activity after 30 minutes is only insignificantly lower than after an hour's incubation, and then it does not change to any significant extent for four hours.

On completion of incubation the small flasks received additionally 0.44 ml of 9.3 N solution of $HClO_4$ for every 10 ml of suspension, samples were cooled in an ice bath for 30 minutes and then nucleoproteids were separated by a previously described technic /7,8/. Fractionation of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) was done by two methods: by a somewhat revised Odzhur method /7,8/ and by a combined method. In the combined method fractionation was done as follows: 100 mg weighed portions of dry nucleic acids were hydrolyzed in centrifuge tubes with 3 ml of 0.5N KOH solution at 37° for 18 hours, after which the tubes were cooled in an ice bath and DNA together with albumins were precipitated with perchloric acid (final concentration 3%). After standing for 30 minutes in an ice bath the fluid containing RNA and the hydrolysate of the active labile fraction of albumin was separated by centrifuging, and from the precipitate, following triple washing by 3% $HClO_4$ solution in the cold, the DNA was extracted /7,8/.

The specific activity of RNA and DNA fractions were determined as described earlier /7/, and were expressed in impulses per minute (deduction for background radiation) with conversion to 10 mg of dry nucleoproteids. DNA and RNA content were determined by the diphenylamine and orcin reactions and were expressed in percent of the dry substance. Mean values of the two parallel determinations are presented in the tables. A deviation of up to 10% between parallel probes was allowed.

In <u>in vitro</u> experiments with solid tumors the same amount of buffer and other ingredients per gram gross tissue weight was added as was the case with ascites cells.

EXPERIMENTAL RESULTS AND THEIR DISCUSSION

A number of investigators /1 and others/ pointed out the advantage of conducting experiments with ascites cells <u>in vitro</u> in ascitic fluid and the necessity of adding extracts or homogenates of pigeon or

mouse liver. In the first series of experiments we elucidated the optimal conditions for conducting in vitro experiments with ascites cells of Ehrlich. Results of one of the typical experiments of this series are presented in table 1.

Table 1

Specific activity of NP, DNA, RNA and Content of NA in Ascites Cells of Ehrlich Under Various Conditions of Incubation

Composition of Sample	Specific activity					% content			
	NP	RNA		DNA		RNA		DNA	
		I	II	I	II	I	II	I	II
Ascites cells plus ascitic fluid	61	13		30		2.7			
Ascites cells plus buffer plus liver homogenate	192	21	140	79	55	6.8	7.4	4.6	4.4
Ascites cells plus buffer plus homogenate plus glucose plus nicotinamide	541	37	400	218	142	7.8	7.6	4.8	4.6
Ascites cells plus buffer plus glucose plus nicotinamide	535	31	383	173	138	7.8	8.0	4.7	4.5

Note. I is Odzhur method; II is combined method.

From table 1 it is evident that the lowest specific activity of NP (nucleoproteid) was produced in the variant in which biosynthesis was performed in ascitic fluid. Addition of nicotinic acid amide considerably increases the incorporation of formate-C^{14} in NP and NA.

In vitro experiments on the study of formate-C^{14} incorporation in ascites cells of Ehrlich are most expeditiously conducted in a medium consisting of a buffer with addition of glucose and nicotinamide. And this medium was further employed.

In comparing the specific activity of DNA fractions and especially of RNA, separated by the different methods (Odzhur and combined), a sharp difference is seen, since RNA and DNA content differ only insignificantly (by 2.5-8%). The RNA fraction, separated by the combined method (hydrolysis with 0.5N KOH solution), was 7-12 times more active than that separated by the Odzhur method (hydrolysis with $HClO_4$).

The DNA fraction, on the contrary, was more active in the separation by the Odzhur method. Results of this and many other experiments (see table 3), in which the specific activity of DNA and RNA fractions, separated by different methods, were at sharp variance while the RNA and DNA content were approximately identical, probable indicate the presence in ascites cells of some substance (or substances) actively incorporating formate, and with bonds labile to nucleoproteids of non-NA but apparently albumin nature. This substance hydrolyzes readily with 0.5N KOH solution and is shown in the RNA fraction on separation of NA by the combined method. With hydrolysis in the cold with $HClO_4$ by the Odzhur method it is not cleaved and does not contaminate the RNA fraction but then hydrolyzes partially in the separation of DNA at 80° and is reflected in the specific activity of this fraction. On hydrolysis this active substance disintegrates into low molecular fragments, producing a burette reaction, but not precipitating with alcohol, trichloroacetic and perchloric acids.

In table 2 results are presented which were derived with solid tumor of Ehrlich and other mouse tissue in an in vivo experiment. Two groups of animals were employed: control and dopan-treated.

Table 2

Specific Activity and RNA and DNA Content in Solid Ehrlich Tumor and Certain Tissues of Control and Dopan-Treated Mice (formate-C^{14} introduced in vivo)

Object of study		Specific activity				% content			
		RNA		DNA		RNA		DNA	
		I	II	I	II	I	II	I	II
Tumor	Control..	59	110	52	52	3.1	3.1	5.0	5.0
	Dopan....	104	128	60	60	4.0	4.0	6.7	6.2
Spleen	Control..	182	269	387	682	5.2	5.2	16.8	
	Dopan....	232	385	455	766	5.7	5.7	15.8	13.8
Liver	Control...	37	124	51	42	6.2	6.2	4.3	3.3
	Dopan...	40	116	51	44	5.9	5.9	3.8	3.4
Small intestine	Control plus Dopan...	246	402	174	178	6.4	6.0	10.0	10.0

Note. I is Odzhur method; II is combined method.

It is evident from table 2 that approximately identical amounts of RNA were determined by the different methods. However the content of DNA by the combined method in most cases was slightly lowered, possibly due to labile DNA /5/. The specific activity of RNA derived by the combined method is in all tissues higher than in the RNA separated by the Odzhur method. The specific activity of the DNA fraction of the tumor and small intestine derived by the one or the other method

is identical, in the liver slightly higher in the Odzhur method, but this can be caused by a somewhat more complete extraction of DNA from the liver by the Odzhur method. A strongly increased specific activity of DNA of the spleen, fractionated by the "combined" method is apparently caused by the presence of some substances actively incorporating formate, which are more easily hydrolyzed by hot perchloric acid than by a 0.5N solution of KOH.

It is evident from the data in tables 1 and 2 that the readily alkalihydrolyzed fraction intensively incorporating formate is contained in various tissues, but in relatively lesser quantities than in ascites cells.

The Odzhur method is not suited for the determination of specific activity of RNA and DNA of ascites cells, although it is quite acceptable for work with solid tumors and tissues of mice and rats. Altogether unacceptable are the methods according to which RNA is extracted in alkali hydrolysis. In fractionating RNA from ascites cells by the Odzhur method the labile active albumin fraction does not remain detached from nucleoproteids, but is partially hydrolyzed in the further extraction of DNA by perchloric acid at 80°. In extraction of RNA by the combined method, the active labile fraction is simultaneously hydrolyzed, but the DNA is not contaminated.

The Odzhur determination of RNA (in two weighed portions of 50-100 mg of dry nucleoproteids) DNA determination by the combined method (in two different weighed portions) cumbersomely requires a large quantity of material. We made the assumption that it is possible to extract from one and the same weighed portions the RNA by the Odzhur method, to then hydrolyze the active labile fraction with KOH, and to extract DNA by the Odzhur method from the residue of albumin and DNA separated from the hydrolysate. To elucidate this possibility a number of experiments were conducted which disclosed that after processing one and the same weighed portion at first with perchloric acid and then with KOH, the DNA partially passes into a non-precipitable form, partially disintegrates, and appears in the final fraction only in $\frac{1}{2}$-$\frac{1}{4}$ of the initial quantity.

Also unacceptable was the lowering of the KOH concentration to 0.25N, since here part of the labile fraction passes into the RNA fraction and part into the DNA fraction. Experiments were performed for the extraction of RNA and DNA with lower concentrations of perchloric acid. It turned out that in fractionation with 1N and 1.2N $HClO_4$ up to 20-30% of the RNA remained unextracted (6 analogous experiments with different ascites). In extraction of DNA with 0.5N $HClO_4$ solution the DNA was completely separated, but at the same time nonetheless some quantities of the hydrolysate of the labile albumin were also extracted, which disrupted the determination of the specific activity of DNA

In further work the specific activity of RNA was determined by the Odzhur method, and DNA in two other weighed portions - by the combined method. The difference in activity of RNA separated by the combined method and by the Odzhur method represents the activity of the hydrolysate of the labile albumin fraction.

In table 3 the results are presented for the comparison of in vivo and in vitro formate-C^{14} incorporation into ascites cells and solid Ehrlich tumor (in vivo). It should be noted that in the solid Ehrlich tumor and in the Crocker sarcoma in in vitro experiments the inclusion of formate was very insignificant, and sometimes was altogether without evidence, for which reason the results of these experiments are not presented

Table 3

Specific Activity and NA Content in Ehrlich Carcinoma

Object of investigation	Conditions of experiment	Specific activity imp/min per 10 mg				Content (%)				Specific activity of RNA / Specific activity of DNA			
		RNA		DNA		RNA		DNA		found	arithmetic mean	error t	reliability of difference
		I	II	I	II	I	II	I	II				
Ascites	in vivo	121	205	78	43	9.4	8.2	5.7	5.0	2.8			
		87	209	80	66	8.6	7.5	5.8	5.3	1.3			
		43	112	55	23	7.1	6.9	5.4	5.4	1.9	1.9	±0.32	
		52	148	60	33	8.4	8.4	6.8	6.0	1.6			
	in vitro	17	188	139	52	7.6	7.6	5.7	5.0	0.3			T=4.
		13	210	107	59	9.5	8.2	5.3	5.3	0.25	0.31	±0.034	
		10	62	104	36	6.9	6.8	5.8	4.7	0.3			
		23	273	140	81	8.6	0.1	6.8	6.3	0.4			
Subcutaneous tumor	in vivo	92	193	73	68	4.3	4.7	5.2	4.5	1.4			
		47	92	41	39	3.7	3.8	5.1	4.7	1.2	1.30	±0.115	
		86	139	63	62	3.6	3.6	5.3	5.3	1.4			
		59	110	52	52	3.1	3.1	5.0	5.0	1.2			

Note. I is Odzhur method; II is combined method

It is evident from table 3 that in most cases somewhat more RNA and DNA is extracted by the Odzhur method than by the combined method but fundamentally the difference does not exceed 10-15%. In contrast, in some cases a higher content of NA is determined by the combined method. And in these experiments a sharp increase of the specific activity of RNA was disclosed in fractionation by the combined method due to hydrolysis of the active labile albumin fraction. In experiments with ascites cells in vitro the specific activity of

the RNA fraction by the combined method was 4-6 times higher than by the Odzhur method, and in in vivo experiments - 2-3 times higher. In in vivo tests with solid tumor the radioactivity of RNA separated by the combined method was 1½-2 times higher than by the Odzhur method. In the solid tumor the specific activity of DNA by the Odzhur and combined method was approximately identical. In experiments with ascites cells the separation of DNA by the Odzhur method disclosed a contamination by the hydrolysate of the highly active albumin fraction.

By comparing intensive formate-C^{14} incorporation into RNA (fractionated by the Odzhur method) and in DNA (fractionated by the combined method) in in vivo and in vitro experiments it is seen that formate inclusion into RNA is sharply depressed in in vitro experiments.

Analogous results are produced even in experiments with ascites and solid tumor of Crocker sarcoma. In certain experiments with ascites cells in vitro the ratio of specific activity of RNA to activity of the hydrolysate of the labile albumin fraction approached 1: 25, that is, the albumin fraction in these experiments was relatively greater than in experiments in vitro with ascites cells of Ehrlich. The ratio of specific activity of RNA and DNA in ascites cells of Crocker sarcoma in experiments in vivo was 2.4 0.5, in vitro - 0.2 0.07, in solid tumor in experiments in vivo - 1.1.

CONCLUSIONS

1. In ascites cells of Ehrlich and of Crocker sarcoma a labile substance (or group of substances) was discovered, apparently of albumin nature, actively incorporating formate-C^{14}, readily hydrolyzed in alkali in the cold and more stable to perchloric acid. In experiments in vitro with ascites cells of Ehrlich and of Crocker sarcoma the specific activity of the hydrolysate of this substance was 6-25 times higher than that of RNA and in experiments in vivo - 2-3 times higher.

2. In the fractionation of RNA and DNA from nucleoproteids of ascites cells, the RNA should be extracted in the cold with 1.7N solution of $HClO_4$, and the DNA should be precipitated from alkaline hydrolysates of parallel weighed portions with albumins, and following separation and washing of residues the DNA should be extracted from the latter with 0.5N solution of $HClO_4$ at 80° by the Odzhur method.

3. By comparing the degree of formate-C^{14} incorporation into nucleic acids of ascites cells in vitro and in vivo a sharp depression of incorporation into RNA in experiments in vitro is discovered.

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